6. The method of claim 4, wherein the alkyl phosphonate internucleotide linkage is a methylphosphonate internucleotide linkage.

7. The method of claim 1, wherein the oligonucleotide comprises about from 15 to 25 nucleotides.

8. The method of claim 1, wherein the oligonucleotide is complementary to a gene of a virus, pathogenic organism, or a cellular gene.

9.(Renumbered)The method of claim 1, wherein the oligonucleotide is complementary to a gene of a virus involved in a disease selected from the group consisting of AIDS, oral and genital herpes, papilloma warts, influenza, foot and mouth disease, yellow fever, chicken pox, shingles, adult T-cell leukemia, Burkitt's lymphoma, nasopharyngeal carcinoma, and hepatitis.

10.(Renumbered)The method of claim 1, wherein the oligonucleotide is complementary to a gene encoding a protein associated with Alzheimer's disease.

11.(Renumbered)The method of claim 1 wherein the oligonucleotide is complementary to a gene encoding a protein in a parasite causing a parasitic disease selected from the group consisting of amebiasis, Chagas' disease, toxoplasmosis, pneumocytosis, giardiasis, cryptoporidiosis, trichomoniasis, malaria, ascariasis, filariasis, trichinosis, schistosomiasis infections.

REMARKS

Claims 1-8 and 12-14 were originally filed with this application. Applicant has renumbered claims 12-14 as claims 9-11. Accordingly, the dependency of renumbered claim 9 has also been amended. Therefore, claims 1-11 are currently pending in this application. Claim 1 has been amended to more clearly recite that which applicants claims as their invention. This amendment contain no new matter, as support therefor is found in the specification at page 9, lines 15 to 29. A copy of the amendments made to the claims follow this preliminary amendment.

In addition, the specification has been amended to correct obvious typographical errors. In particular, the error at page 12, line 14, referring to "oligonucleotide 1 (SEQ ID NO:10)", is obvious in view of Table 1, at page 22, which shows that oligonucleotide 11 has the sequence set forth as SEQ ID NO: 10. Accordingly, no new matter has been introduced by way of these amendments. A copy of the amendments made to these paragraphs follows preliminary amendment.

Please charge our Deposit Account No. 08-0219 the basic Filing Fee of $355. The fee charged reflects the applicant's small entity status. In addition, please charge any fees or credit any overpayment to this account.

Respectfully submitted,

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Marked Up Version of Replacement Paragraphs in Specification Under 37 C.F.R. §1.121 (b)(1)(iii)

In the Specification:

Paragraph on page 5, lines 1-25:

Several preliminary studies on this topic have been published. Agrawal et al. *(Proc. Natl. Acad. Sci. (USA)* (1991) **88:**7595-7599) describes the [intravenously] intravenous and [intraperitoneally] intraperitoneal administration to mice of a 20mer phosphorothioate linked-oligonucleotide. In this study, approximately 30% of the administered dose was excreted in the urine over the first 24 hours with accumulation preferentially in the liver and kidney. Plasma half-lives ranged from about 1 hour ($t_{1/2\alpha}$) and 40 hours ($t_{1/2\beta}$), respectively. Similar results have been reported in subsequent studies (Iversen (1991) *Anti-Cancer Drug Design* **6:**531-538; Iversen (1994) *Antisense Res. Devel.* **4:**43-52; and Sands (1994) *Mol. Pharm.* **45:**932-943). However, stability problems may exist when oligonucleotides are administered intravenously and intraperitoneally. More recently, Agrawal et al. reported that oligonucleotide hybrids containing 2'-O-methyl ribonucleotides at both the 3'- and 5' ends and deoxyribonucleotide phosphorothioates in the interior portion were absorbed through the gastrointestinal (GI) tract of rats (*Biochem. Pharm.* (1995) **50**:571-576).

Paragraph at page 7, line 31 to page 8, line 12:

The term "non-phosphodiester-linkages" as used herein refers to a synthetic covalent attachment between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' nucleotide phosphate has been replaced with any number of chemical groups. Preferable synthetic linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, carbamates, carbonates, phosphate

triesters, acetamidate, and carboxymethyl esters. In one preferred embodiment of the invention, [the] all of the nucleotides of the oligonucleotide [comprises] are linked via phosphorothioate and/or phosphorodithioate linkages.

Paragraph on page 9, lines 15-28:

For purposes of the invention, the term "2'-substituted oligonucleotide" refers to an oligonucleotide having a sugar attached to a chemical group other [that] than a hydroxyl group at its 2' position. The 2'-OH of the ribose molecule can be substituted with -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms, e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl (such as a 2'-O-methyl), 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups.

Paragraph at page 9, line 30 to page 10, line 22:

In one preferred embodiment of the invention, the oligonucleotide administered includes at least one 2'-substituted ribonucleotide at its 3' terminus. In some embodiments, all but four or five nucleotides at its 5' terminus are 2'-substituted ribonucleotides, and in some embodiments, these four or five unsubstituted 5' nucleotides are deoxyribonucleotides. In other embodiments, the oligonucleotide has at least one 2'-substituted ribonucleotide at both its 3' and 5' termini, and in yet other embodiments, the oligonucleotide is composed of 2'-substituted ribonucleotides in all positions with the exception of at least four or five contiguous deoxyribonucleotide nucleotides in any interior position. Another aspect of

the invention includes the administration of an oligonucleotide composed of nucleotides that are all 2'-substituted ribonucleotides. Particular embodiments include oligonucleotides having a 2'-O-alkyl-ribonucleotide such as a [2'-0 methyl] 2'-O-methyl. Other embodiments include the administration of chimeric oligonucleotides. In one preferred embodiment, the chimeric oligonucleotide has at least one alkylphosphonate internucleotide linkage at both its 3' and 5' ends and having phosphorothioate internucleotide linkages.

Paragraph on page 11, lines 15-17:

In another embodiment, the oligonucleotide is complementary to a gene encoding a protein [in] associated with Alzheimer's disease.

Paragraph on page 12, lines 10-14:

FIG. 1 is a graphic representation showing the time course of radiolabelled oligonucleotide in liver, kidney and plasma following the oral administration of radiolabelled phosphorothioate (PS) oligonucleotide [1] 11 (SEQ ID NO:10);

Paragraph on page 19, lines 17-32:

The oligonucleotides administered to the animal may be hybrid oligonucleotides in that they contain both deoxyribonucleotides and at least one 2' substituted ribonucleotide. For purposes of the invention, the term "2'-substituted" means substitution at the 2' position of the ribose with, e.g., a -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl, 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl,

cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups. Useful substituted ribonucleotides are [2'-0-alkyls] 2'-O-alkyls such as [2'-0-methyl] 2'-O-methyl.

Paragraph at page 35, line 32 to page 36, line 18:

The chemical form of radioactivity in rat plasma was further evaluated by HPLC as shown [is] in FIG. 4A and 4B, demonstrating the presence of both intact PS oligonucleotide (A) as well as metabolites (B) 12 hours after oral administration (see FIG. 4B). Intact oligonucleotide was also detected in rat liver 6 hours (FIG. 5B) and 12 hours (FIG. 5C) after oral administration. Radioactivity in rat brain, thymus, heart, lung, liver, kidney, adrenals, stomach, small intestine, large intestine, skeletal muscle, testes, thyroid, epidermis, whole eye, and bone marrow was detectable 48 hours after oral administration of the radiolabelled oligonucleotide. For unmodified oligonucleotide, minimal intact form was detectable in rat tissue samples. However, as shown in FIG. 11A for the hybrid oligonucleotide and in FIG. 11B for the chimeric oligonucleotide, intact oligonucleotides were detected in plasma and tissue samples of the liver, kidney, spleen, heart, and lung.

Paragraph on page 38, lines 20-26:

Oral absorption of oligonucleotides in fasting animals was also determined with PS- oligonucleotide and hybrid oligonucleotide. Decreased absorption rates were found, indicating that the retention time of the oligonucleotides in the gastrointestinal tract in the fasting animals may be lower [that] than in non-fasting animals.

Paragraph on page 39, lines 21-31:

An unmodified HIV-specific 25mer oligonucleotide and hybrid 25mer phosphorothioate-linked oligonucleotide having SEQ ID NO:10 and containing [2'-0-methyl] 2'-O-methyl ribonucleotide 3' and 5' sequences and a deoxyribonucleotide interior, as well as two hybrid 18mer phosphorothioate-linked oligonucleotides having SEQ ID NOS:20 and 21, and containing [2'-0-methyl] 2'-O-methyl ribonucleotide 3' and 5' sequences and a deoxyribonucleotide interior, were synthesized, purified, and analyzed as follows.

Amendments Made to the Claims

1.(Amended)A method for introducing an intact oligonucleotide into a mammal, the method comprising the step of orally administering to the mammal a chimeric oligonucleotide, the oligonucleotide comprising about 6 to 50 nucleotides linked via at least one phosphorothioate internucleotide linkage and at least one internucleotide linkage selected from the group consisting of alkylphosphonate, phosphorodithioate, alkylphosphonothioate, phosphoramidate, phosphoramidite, phosphate ester, carbamate, carbonate, phosphate triester, acetamidate, and carboxymethyl ester, the oligonucleotide further comprising at least one 2'-O-alkyl ribonucleotide, whereby the oligonucleotide is present in intact form in plasma at least six hours following oral administration.

9[12].(Amended)The method of claim [11] 1 wherein the oligonucleotide is complementary to a gene of a virus involved in a disease selected from the group consisting of AIDS, oral and genital herpes, papilloma warts, influenza, foot and mouth disease, yellow fever, chicken pox, shingles, adult T-cell leukemia, Burkitt's lymphoma, nasopharyngeal carcinoma, and hepatitis.

10[13].(Renumbered)The method of claim 1, wherein the oligonucleotide is complementary to a gene encoding a protein associated with Alzheimer's disease.

11[14].(Renumbered)The method of claim 1 wherein the oligonucleotide is complementary to a gene encoding a protein in a parasite causing a parasitic disease selected from the group consisting of amebiasis, Chagas' disease, toxoplasmosis, pneumocytosis, giardiasis, cryptoporidiosis, trichomoniasis, malaria, ascariasis, filariasis, trichinosis, schistosomiasis infections.

APPLICATION FOR
UNITED STATES LETTERS PATENT
IN THE
UNITED STATES PATENT AND TRADEMARK OFFICE

(Case No. HYZ-030CIP)

Title:

A METHOD OF DOWN-REGULATING GENE EXPRESSION

Inventors:

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Robert B. Diasio
and
Ruiwen Zhang

Assignee:

HYBRIDON, INC.
A Corporation of the State of Delaware

A METHOD OF DOWN-REGULATING GENE EXPRESSION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of copending Patent Application Serial No. 08/709,910, filed September 10, 1996, which is a continuation-in-part of copending Patent Application Serial No. 08/328,520, filed October 25, 1994.

BACKGROUND OF THE INVENTION

The present invention relates to the control of gene expression. More particularly, this invention relates to the use of synthetic, modified oligonucleotides to down-regulate the expression of a gene in an animal.

The potential for the development of an antisense oligonucleotide therapeutic approach was first suggested in three articles published in 1977 and 1978. Paterson et al. (*Proc. Natl. Acad. Sci. (USA)* (1977) **74:**4370-4374) discloses that cell-free translation of mRNA can be inhibited by the binding of an oligonucleotide complementary to the mRNA. Zamecnik et al. (*Proc. Natl. Acad. Sci. (USA)* (1978) **75:**280-284 and 285-288) discloses that a 13mer synthetic oligonucleotide that is complementary to a part of the Rous sarcoma virus (RSV) genome inhibits RSV replication in infected chicken fibroblasts and inhibits RSV-mediated transformation of primary chick fibroblasts into malignant sarcoma cells.

These early indications that synthetic oligonucleotides can be used to inhibit virus propagation and neoplasia have been followed by the use of synthetic oligonucleotides to inhibit a wide variety of viruses, such as HIV (see, e.g., U.S. Patent No. 4,806,463); influenza (see, e.g., Leiter et al. (1990) (*Proc. Natl. Acad. Sci. (USA)* **87:**3430-3434); vesicular stomatitis virus (see, e.g., Agris et al. (1986) *Biochem.* **25:**6268-6275); herpes simplex (see, e.g., Gao et al. (1990) *Antimicrob. Agents Chem.* **34:**808-812); SV40 (see, e.g., Birg et al. (1990) (*Nucleic Acids Res.* **18:**2901-2908); and human papilloma virus (see, e.g., Storey et al. (1991) (*Nucleic Acids Res.* **19:**4109-4114). The use of synthetic oligonucleotides and their analogs as antiviral agents has recently been extensively reviewed by Agrawal (*Trends in Biotech.* (1992) **10:**152-158).

In addition, synthetic oligonucleotides have been used to inhibit a variety of non-viral pathogens, as well as to selectively inhibit the expression of certain cellular genes. Thus, the utility of synthetic oligonucleotides as agents to inhibit virus propagation, propagation of non-viral, pathogens and selective expression of cellular genes has been well established.

Improved oligonucleotides have more recently been developed that have greater efficacy in inhibiting such viruses, pathogens and selective gene expression. Some of these oligonucleotides having modifications in their internucleotide

linkages have been shown to be more effective than their unmodified counterparts. For example, Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1988) **85:**7079-7083) teaches that oligonucleotide phosphorothioates and certain oligonucleotide phosphoramidates are more effective at inhibiting HIV-1 than conventional phosphodiester-linked oligodeoxynucleotides. Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1989) **86:**7790-7794) discloses the advantage of oligonucleotide phosphorothioates in inhibiting HIV-1 in early and chronically infected cells.

In addition, chimeric oligonucleotides having more than one type of internucleotide linkage within the oligonucleotide have been developed. Pederson et al. (U.S. Patent Nos. 5,149,797 and 5,220,007 discloses chimeric oligonucleotides having an oligonucleotide phosphodiester or oligonucleotide phosphorothioate core sequence flanked by nucleotide methylphosphonates or phosphoramidates. Furdon et al. (*Nucleic Acids Res.* (1989) **17:**9193-9204) discloses chimeric oligonucleotides having regions of oligonucleotide phosphodiesters in addition to either oligonucleotide phosphorothioate or methylphosphonate regions. Quartin et al. (*Nucleic Acids Res.* (1989) **17:**7523-7562) discloses chimeric oligonucleotides having regions of oligonucleotide phosphodiesters and oligonucleotide methylphosphonates. Inoue et al. (*FEBS Lett.* (1987) **215:**237-250) discloses chimeric oligonucleotides

having regions of deoxyribonucleotides and 2'-O-methyl-ribonucleotides.

Many of these modified oligonucleotides have contributed to improving the potential efficacy of the antisense oligonucleotide therapeutic approach. However, certain deficiencies remain in the known oligonucleotides, and these deficiencies can limit the effectiveness of such oligonucleotides as therapeutic agents. For example, Wickstrom (*J. Biochem. Biophys. Meth.* (1986) **13**:97-102) teaches that oligonucleotide phosphodiesters are susceptible to nuclease-mediated degradation, thereby limiting their bioavailability *in vivo*. Agrawal et al. (*Proc. Natl. Acad. Sci. (USA)* (1990) **87**:1401-1405) teaches that oligonucleotide phosphoramidates or methylphosphonates when hybridized to RNA do not activate RNase H, the activation of which can be important to the function of antisense oligonucleotides. Thus, a need for methods of controlling gene expression exists which uses oligonucleotides with improved therapeutic characteristics.

Several reports have been published on the development of phosphorothioate-linked oligonucleotides as potential anti-AIDS therapeutic agents. Although extensive studies on chemical and molecular mechanisms of oligonucleotides have demonstrated the potential value of this novel therapeutic strategy, little is known about the pharmacokinetics and metabolism of these compounds *in vivo*.

Several preliminary studies on this topic have been published. Agrawal et al. *(Proc. Natl. Acad. Sci. (USA)* (1991) **88:**7595-7599) describes the intravenously and intraperitoneally administration to mice of a 20mer phosphorothioate linked-oligonucleotide. In this study, approximately 30% of the administered dose was excreted in the urine over the first 24 hours with accumulation preferentially in the liver and kidney. Plasma half-lives ranged from about 1 hour ($t_{1/2\alpha}$) and 40 hours ($t_{1/2\beta}$), respectively. Similar results have been reported in subsequent studies (Iversen (1991) *Anti-Cancer Drug Design* **6:**531-538; Iversen (1994) *Antisense Res. Devel.* **4:**43-52; and Sands (1994) *Mol. Pharm.* **45:**932-943). However, stability problems may exist when oligonucleotides are administered intravenously and intraperitoneally. More recently, Agrawal et al. reported that oligonucleotide hybrids containing 2'-O-methyl ribonucleotides at both the 3'- and 5' ends and deoxyribonucleotide phosphorothioates in the interior portion were absorbed through the gastrointestinal (GI) tract of rats (*Biochem. Pharm.*(1995) **50**:571-576).

Thus, there remains a need to develop more effective therapeutic methods of down-regulating the expression of genes which can be easily manipulated to fit the animal and condition to be treated, and the gene to be targeted. Preferably, these methods should be simple, painless, and precise in effecting the target gene.

SUMMARY OF THE INVENTION

The present invention provides a method of down-regulating the expression of a gene in an animal which involves the administration of an oligonucleotide complementary to the gene via an oral route, thereby bypassing the complications which may be experienced during intravenous and other modes of in vivo administration.

It has been discovered that hybrid oligonucleotides with other than phosphodiester bonds and having at least one 2'-substituted ribonucleotide and chimeric oligonucleotides with at least two different types of internucleotide linkages are relatively stable *in vivo* following oral administration to an animal, and that these molecules are successfully absorbed from the gastrointestinal tract and distributed to various body tissues. This discovery has been exploited to develop the present invention, which is a method of down-regulating the expression of a gene in an animal.

This method is also a means of examining the function of various genes in an animal, including those essential to animal development. Presently, gene function can only be examined by the arduous task of making a "knock out" animal such as a mouse. This task is difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock out" would

produce a lethal phenotype. The present invention overcomes the shortcomings of this model.

In the method of the invention, a pharmaceutical formulation containing an oligonucleotide complementary to the targeted gene is orally administered in a pharmaceutically acceptable carrier to the animal harboring the gene. The oligonucleotide inhibits the expression of the gene, thereby down-regulating its expression.

For purposes of the invention, the term "animal" is meant to encompass humans as well as other mammals, as well as reptiles amphibians, and insects. The term "oral administration" refers to the provision of the formulation via the mouth through ingestion, or via some other part of the gastrointestinal system including the esophagus.

As used herein, the term "oligonucleotide" is meant to include polymers of two or more nucleotides or nucleotide analogs connected together via 5' to 3' internucleotide linkages which may include any linkages that are known in the antisense art, including non-phosphodiester linkages. Such molecules have a 3' terminus and a 5' terminus.

The term "non-phosphodiester-linkages" as used herein refers to a synthetic covalent attachment between the 5' end of one nucleotide and the 3' end of another nucleotide in which the

5' nucleotide phosphate has been replaced with any number of chemical groups. Preferable synthetic linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters. In one preferred embodiment of the invention, the all of the nucleotides of the oligonucleotide comprises are linked via phosphorothioate and/or phosphorodithioate linkages.

In some embodiments of the invention, the oligonucleotides administered are modified with other than, or in addition to, non-phosphodiester-internucleotide linkages. As used herein, the term "modified oligonucleotide" encompasses oligonucleotides with modified nucleic acid(s), base(s), and/or sugar(s) other than those found in nature. For example, a 3', 5'-substituted oligonucleotide is an oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position).

A modified oligonucleotide may also be one with added substituents such as diamines, cholestryl, or other lipophilic groups, or a capped species. In addition, unoxidized or partially oxidized oligonucleotides having a substitution in one nonbridging oxygen per nucleotide in the molecule are also considered to